Applicant: **ABULJADAYEL**

Serial No.: **09/742,520**

Filed: **December 20, 2000**

Art Unit: **1644**

For: **METHOD OF PREPARING AN UNDIFFERENTIATED CELL**

Examiner: **D. A. Saunders**

DECLARATION UNDER RULE 37 CFR 1.132

I, Dr Illham M. S. S. Abuljadayel DO SOLEMNLY and **SINCERELY DECLARE** as follows:

1. I currently reside at 11 St John's Wood Park, St John's Wood, London, England. I am the inventor of the subject matter in the above-referenced application. I received my Bachelor's degree in cell biology from King's College (London) and my Ph.D., also from King's College, in immunology in 1990.

2. Since 1982, I have been working as a molecular and cellular biologist and thus I have had more than 19 years of experience in cell biology. I have published approximately 14 articles in peer-reviewed journals.

3. I have read and am familiar with the March 6, 2002, Office Action issued in regard to the present application, as well as the specification of the present application (US SN 09/742,520). Thus, from my education, training and experience and familiarity with the present application, I respectfully submit that I am well qualified to speak as to the present application, its disclosure, the knowledge in the art, the practical implications and the technical teachings of the findings set forth in the present application to the skilled artisan.

4. Agents

4.1 Experiment 1

4.1.1 Patients

Blood samples were obtained from patients with B-cell chronic lymphocytic leukaemia (B-CLL), patients with IgA deficiency, patients with HIV infections (HIV+) and patients with various clinical conditions.

The relative and absolute number of B-lymphocytes and MHC class II positive cells in the blood of patients with no B-cell malignancy was extremely low compared to those found in the blood of B-CLL patients.

4.1.2 Treatment of Blood with an Agent

Blood samples, once obtained, were treated with an agent and left to mix on a head to head roller at room temperature for a maximum of 24 hours. Samples were mixed first on a head to head roller for 15 minutes after which they were left to incubate in an incubator at 22°C. The concentration of monoclonal antibody added to blood samples varied from 10-50 µl/ml of blood.

Incubation periods with monoclonal antibodies ranged from 2 hour, 4 hour, 6 hour, 12 hour to 24 hour intervals.

4.1.3 Flow Cytometry

In order to determine the immunophenotypic changes that accompanied different types of treatment by the agents the blood samples were stained with labelled monoclonal antibody to the CD45 marker. The whole blood samples were stained and lysed according to the manufacturer instructions. Flow cytometry analysis was performed on a FACScan $\bar{a}$ with either simultest or PAINT A GATE software (BDIS) which included

negative controls back tracking. 10,000 to 20,000 events were acquired and stored in list mode files.

4.1.4 Morphology

Morphology was analysed using microscopy and Wright's stain.

4.1.5 Agent

The agent was monoclonal antibody to the homologous region of the beta chain of the HLA-DR and the effect of the agent on the cell marker CD45 was analysed by flow cytometry.

4.1.6 Results

Engagement of the β-chain of the DR antigens in treated blood samples including those obtained from patients with B cell chronic lymphocytic leukaemia (B-CLL) showed that such a treatment affects the level of CD45 antigens on B-lymphocytes. The overall immunophenotypic changes that took place on stimulation of the β-chain of the DR antigen gave rise to different types of cells that could be segregated on the basis of the level of CD45 expression as well as morphology as determined by forward scatter and side scatter (size and granulity respectively) and these results are presented in Table A.

Table A. Immunophentyping of patients with B-CLL and other conditions before (B) and after (A) treatment of blood with monoclonal antibody to the homologous region of the beta chain of the HLA-DR with monoclonal antibodies to CD45.

Patients ID & Diagnosis	% of cells with the marker CD45 low	
	B	A
1 B-CLL	7.5	21.9
2 B-CLL	8.8	38.3
3 B-CLL	9.9	33.8
4 B-CLL	2.1	7
5 B-CLL	34.9	9.4
7 B-CLL	45.6	13.9
8 B-CLL	71.1	34.5
10 B-CLL	16.3	48
92 out patient	27.4	26.6
91 out patient	40.4	44.3
87 out patient	36.1	28.7
21 out patient	44.3	47.6
34 out patient	54.8	59.6
39 out patient	30.5	42.1
HIV+	66.8	63.5
IgA Deficient	41.9	33.3

On treatment the relative number of CD45 low cells (when compared to untreated samples) increased significantly.

Samples 5, 7 and 8 revealed opposite immunophenotypic changes to those obtained with other samples obtained from other B-CLL patients. This is because these samples were analysed at a much earlier incubation time with the monoclonal antibody. In fact sequential analysis of blood samples after treatment showed that the immunophenotypic

changes undertaken by B lymphocytes were time dependent. Thus the immunophenotypic changes measured at time X will not be the same at time X plus.

The effect of treatment of blood samples from other patients with no B-cell malignancy showed variable changes in the immunophenotype of cells, because B-lymphocytes are present in a lower amount. However, treatment of enriched fractions of B-lymphocytes obtained from healthy blood donors showed similar immunophenotypic changes to those obtained with B-CLL with high B lymphocyte counts.

4.2 Experiment 2

Patients, Treatment of Blood with an Agent, Flow Cytometry, and Morphology were the same as disclosed in 4.1.1-4.1.4.

4.2.1 Agent

The agent was either
(i) monoclonal antibody to the homologous region of the beta chain of the HLA-DR.
(ii) monoclonal antibody to the homologous region of the beta chain of the HLA-DR antigen and cyclophosphoamide or,
(iii) monoclonal antibody to the homologous region of the class I antigens,
and the effect of the agent on the cell marker CD45 was analysed by flow cytometry.

4.2.2 Results

The results of blood samples treated with monoclonal antibodies to the β- or alpha-chains of the DR antigen or to the β-chain plus cyclophosphoamide or class I antigens are shown in Table B.

Table B. Immunophenotyping of patients with B-CLL before (B) and after treatment of blood with monoclonal antibodies to the homologous region of the alpha-chain of the HLA-DR (AA), the homologous region of the beta-chain of the HLA-DR (AB), monoclonal antibodies to the homologous region of the beta-chain plus cyclophosphoamide (ABC) and the homologous region of class I antigens (AI) measured with time.

	% of cells with the marker CD45+Low				
Patients ID & diagnosis Time	B	AA	AB	ABC	AI
5/6 B-CLL 2H	0	0	5	10	0
10 B-CLL 2H	0	N	0	N	0
09 B-CLL 24H	2	N	N	N	1
HIV+ 6H	4	N	3	N	6
IgA Deficiency 6H	2	N	2	N	4

Treatment of blood sample 5/6 (at 2 hours) with monoclonal antibodies to the β-chain of the DR antigen (AB) or with this monoclonal antibody plus cyclophosphoamide (ABC) generated $CD45^{+}$ low cells. Treatment of blood samples of HIV+ and IgA deficient patients with monoclonal antibody to class I antigen (AI) increased the relative number of $CD45^{+}$ low cells when compared to untreated samples or samples treated with monoclonal antibody to the β-chain of the DR antigen (AB).

4.3 Experiment 3

In Annexe A which is also filed with this Declaration, experimental evidence is provided to show that two other and unrelated agents, namely erythropoietin and GM-CSF also result in the conversion of more committed cells to stem cells (specifically CD34+ cells which are always CD45low cell).

4.4 Experiment 4

Treatment of Blood with an Agent, Flow Cytometry and Morphology were the same as disclosed in 4.1.1-4.1.4.

4.4.1 Agent

The agent was either

(i) monoclonal antibody to the alpha chain of MHC class II antigen (TAL.IB5).

(ii) anti CD2 and anti-CD33 antibodies secondarily labelled with anti-mouse coated magnetic beads.

(iii) monoclonal antibody to the beta chain of MHC class II antigen (CR3/43).

4.4.2 Results

The results of blood samples treated with monoclonal antibody to the alpha chain of MHC class II antigen (TAL.IB5), anti CD2 and anti-CD33 antibodies secondarily labelled with anti-mouse coated magnetic beads or monoclonal antibody to the beta chain of MHC class II antigen (CR3/43) are shown in Figure 1.

Leukocytes were gated according to CD45 low expression (upper panel). These CD45 low cells were then analysed according to CD34 and CD38 expression (lower panels). Cells that are CD38 negative CD34+ are more primitive than cells that are CD38 positive CD34+ (more committed stem cells). Treated leukocytes show a

significant increase in the level of cells that are CD45 low and are CD34 positive when compared to control cells that were untreated.

4.5 Conclusion

In regard to the data disclosed above, the agents:

(i) monoclonal antibody to the homologous region of the beta chain of the HLA-DR antigen;

(ii) monoclonal antibody to the homologous region of the beta chain of the HLA-DR antigen with cyclophosphoamide;

(iii) monoclonal antibody to the homologous region of the class I antigens;

(iv) GM-CSF;

(v) erythropoietin;

(iv) monoclonal antibody to the alpha chain of MHC class II antigen (TAL.IB5);

(v) anti CD2 and anti-CD33 antibodies secondarily labelled with anti-mouse coated magnetic beads;

(vi) and monoclonal antibody to the beta chain of MHC class II antigen (CR3/43)

resulted in an increased number of cells with the marker CD45low when the cell population was contacted with the agent and the cells engaging the agent were incubated.

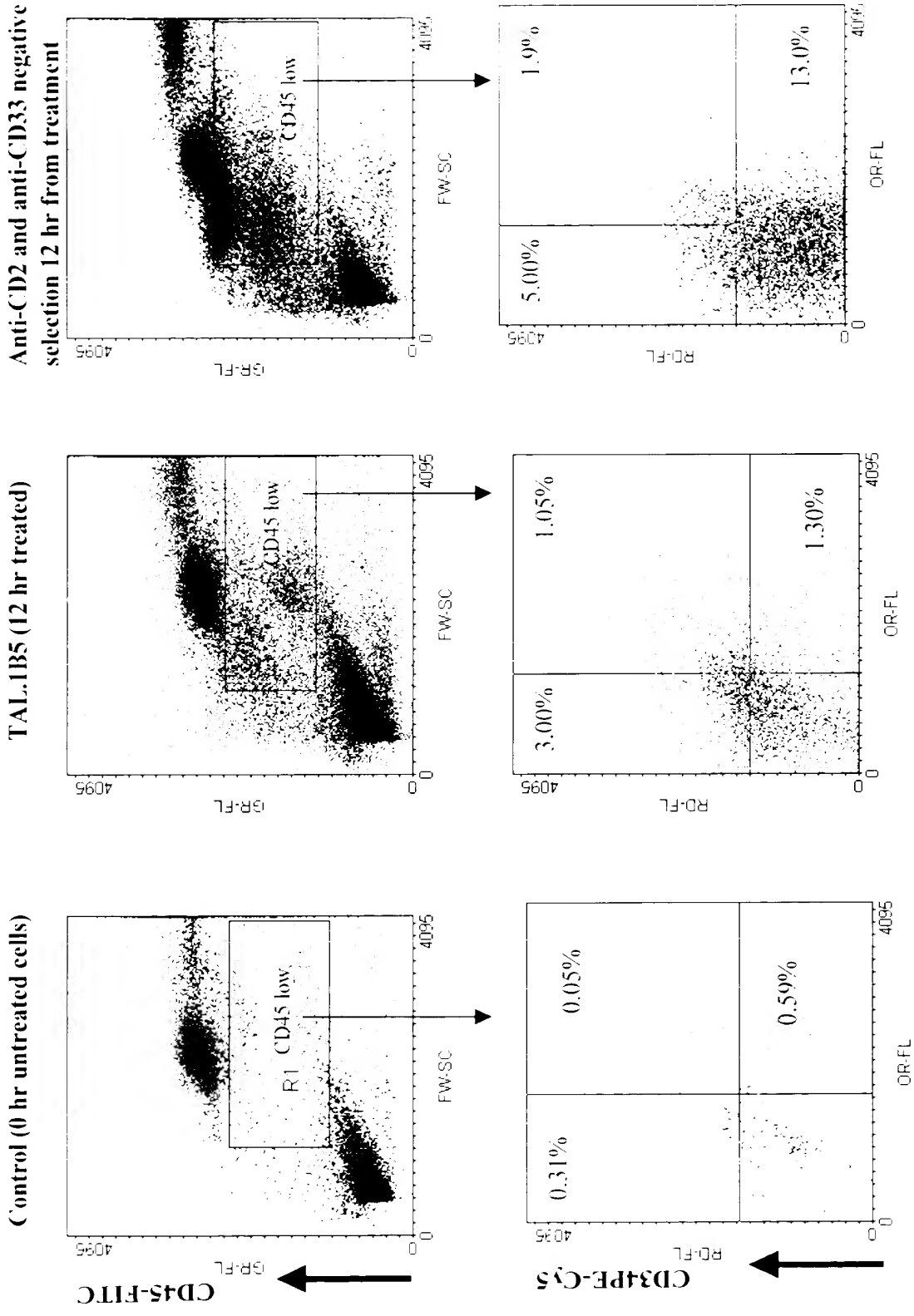
5. Utility

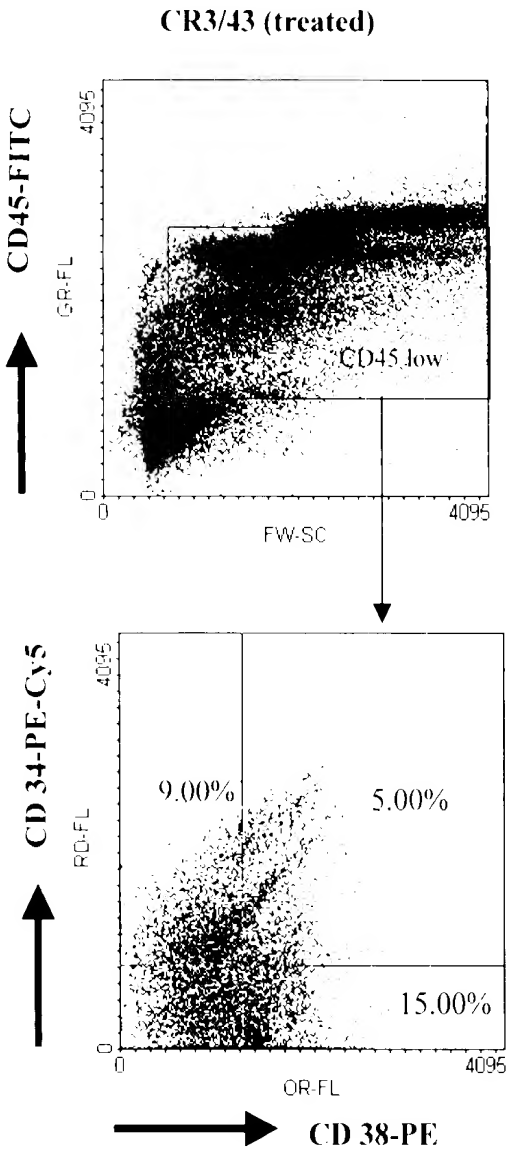
As would have been readily understood at the priority date of the present invention, ie at February 2 1995, CD45low is a marker found on stem cells having a haemopoietic or myeloid nature. A skilled person at the priority date of the above-identified application would have readily understood that undifferentiated cells with CD45low markers could be used in the production of, *inter alia*, more committed haematopoietic cells, for example white blood cells. Thus, CD45low cells could be redifferentiated into more committed haematopoietic cells for instance, and thus a skilled person would have readily understood that the CD45low cells had a utility in the treatment of, for example, leukaemia.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

Signature: 

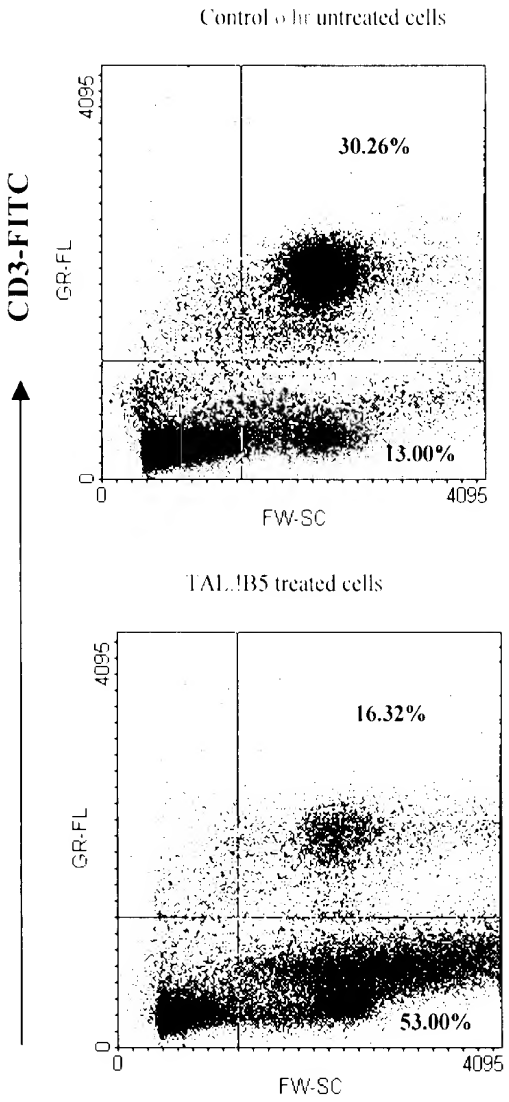
Place: London **Date:** 4. July. 2002



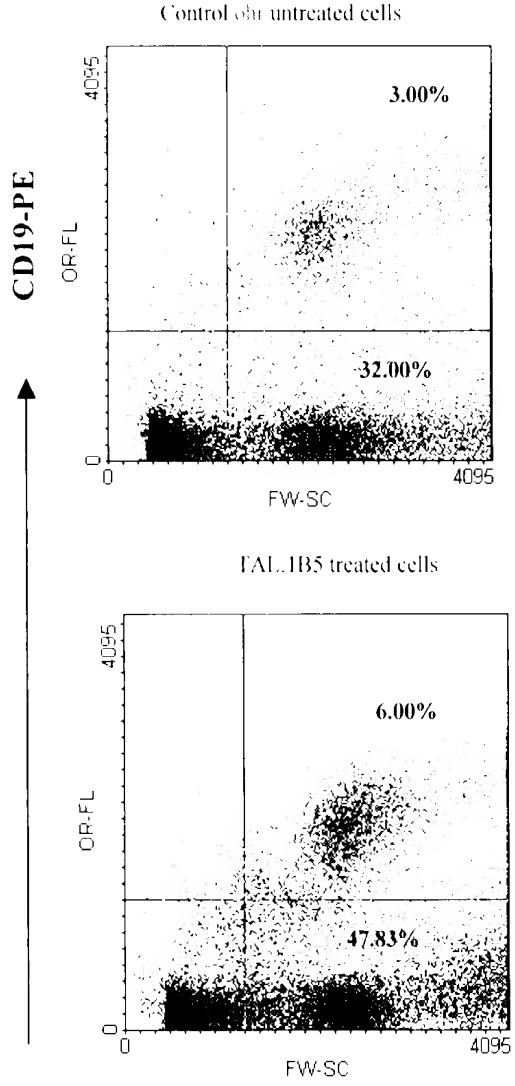


Leucocytes were gated according to CD45 low expression (upper panel). These CD45 low cells were then analysed according to CD34 and CD38 expression (lower panels). As seen below cells that are treated with antibody to the alpha chain of MHC class II antigens (TAL.IB5) or cells subjected to negative selection using anti CD2 and anti-CD33 antibodies secondarily labelled with anti-mouse coated magnetic beads or cells treated with antibody to the beta chain of MHC class II antigens (CR3/43) show low expression of CD45 and are CD34 positive. Those that are CD38 negative CD34+ are more primitive than cells that are CD38 positive CD34+ (more committed stem cells). Treated leucocytes show significant increase in the level of cells that are CD 45 low and are CD34 positive when compared to control cells that were untreated.

Method: Leukocytes were obtained from buffy coat by sedimentation using a density gradient (histopaque). Cells were cultured in Dexter medium containing various agents plus cortisol (a biological response modifier)



CD3 expression on leucocytes before (upper panel) and after (lower panel) treatment with TAL.1B5. The relative number of cells that are CD3 positive decrease from 30.26% to 16.32% on treatment with TAL.1B5. Therefore the relative number of cells that are CD3 negative increase from 13.00% to 53.00% on treatment with TAL.1B5.



CD 19 or HLA-DR expression on leucocytes before (upper panel) and after (lower panel) treatment with TAL.1B5. The relative number of cells that are either CD19 or HLA-DR positive increase from 3% to 6% on treatment with TAL.1 B5. The relative number of cells that are DR or CD19 negative also increase from 32% to 47.83%. These results taken together with the previous diagram show that TAL.1B5 retrodifferentiates T cells (CD3+) to cells that are CD3 negative and are either DR positive or HLA-DR negative .

Annex A – Additional Data

Methods and materials

Nucleated cells and plasma were isolated from buffy coats of healthy blood donors by centrifugation at low speed. Lymphocytes were isolated using Lymphoprep and then enriched using a nylon wool column. B cells were negatively selected (three times) using magnetic beads coated with CD3 and CD33 monoclonal antibodies (specific for the T lymphoid and Myeloid lineage respectively). Cell were suspended in RPM medium containing 50% autologous plasma. Cells cultured in this way were subjected to treatment with either nothing, antibody CR3/43, GM-CSF or erythropoietin. After 6hrs cells were washed and analysed using flow cytometry. The following Panels of Immunostains were used (and are shown in Exhibit Y):

Negative controls to determine nonspecific binding.
CD19 and CD3 for B and T cells respectively.
CD8 and CD4 for cytotoxic and helper T cells respectively.
HLA-DR and CD3 for B cells and activated T cells respectively.
CD34 and CD3 for stem cells and T cells respectively.

Results

As can be seen from Exhibit Y, cells having CD34 cell surface markers appear in cell populations treated with antibody CR3/43, GM-CSF or erythropoietin but not in the untreated control cell population. The results are most striking with the CR3/43 antibody but nonetheless, significant results are seen with GM-CSF and erythopoietin. The appearance of cells having CD34 on their surface is consistent with the appearance of hemopoietic stem cells. The results shown in Exhibit Y confirm that a range of agents may be used to effect the production of undifferentiated cells from more differentiated cells. In other words, Exhibit Y further shows that the invention is operable, in accordance with the teachings in the present application, with agents in addition to CR3/43.

Exhibit Y